AMENDMENT TO THE SPECIFICATION

Please replace the paragraph beginning on page 1, line 5 with the following paragraph:

This application is a divisional application of U.S. Application No. 09/785,866, filed February 16, 2001, which [This application] claims priority to U.S. Provisional Application Serial No. 60/183,078, filed February 16, 2000, entitled "Aspirin-Triggered Lipid Mediators" by Charles N. Serhan and U.S. Provisional Application Serial No. 60/238,814, filed October 6, 2000, entitled "Aspirin-Triggered Lipid Mediators" by Charles N. Serhan, the contents of which are incorporated herein by reference in their entirety.

Please replace the paragraph on page 9 beginning at line 25 with the following amended paragraph:

Figures ~~3~~ 3A-3C depicts ~~conversation~~ of EPA (ω-3) by *B. megaterium* via LC/MS ion chromatogram and mass spectral analysis.

Please replace the paragraph on page 10 beginning at line 15 with the following amended paragraph.

Figures ~~5~~ 5A-5C depicts an LC/MS ion chromatogram of 5,12,18-triHEPE isomers generated from 5*S*, 12*R*-dihydroxy-6*Z*,8*E*,14*Z*,17*Z*-eicosapentaenoic acid (ω-3) by *B. megaterium* and with mass spectral analyses.

Please replace the paragraph on page 11 beginning at line 7 with the following amended paragraph.

Figures ~~8~~ 8A-8D depicts ~~and~~ selected ion monitoring LC/MS/MS chromatograms of monhydroxy products generated from EPA by aspirin acetylated-COX-2 with mass spectral analyses of 18-HEPE, 15-HEPE, and 11-HEPE.

Please replace the paragraph on page 11 beginning at line 28 with the following amended paragraph.

Figures ~~10A~~ 10A-10L depicts LC/MS/MS ion chromatograms and mass spectral analyses of monohydroxy products generated from dihomo-γ-linoleic acid (C20:3, ω-3) and aspirin acetylated-COX-2.

Please replace the paragraph on page 12 beginning at line 9 with the following amended paragraph.

Figures ~~11~~ 11A-11D depict a ~~P~~ proposed ~~S~~ scheme for Generating Functional Arrays of Lipid Signals from ω-3 PUFA via Transcellular Processing Endogenous Inhibitors of Microinflammation. At sites where COX-2 is upregulated and treated with NSAID's, prostaglandin formation from C20:4 is blocked. Systemic ω-3 PUFA are converted via a COX-2NSAID lipoxygenase-type mechanism that stereospecific hydrogen abstraction at (Panel A) C16 or C13 in EPA (C20:5) to give *R* insertions of molecular O_2 to yield 15*R*-H(p)EPE or 18*R*-H(p)EPE from epoxides or are reduced to alcohols or similarly at (Panel B) C13 or C17 in DHA (C22:6) to give insertions of molecular O_2 to yield 13-hydrosy-DHA or 17-hydroxy-DHA. The complete stereochemistry of the trihydroxy- compounds remain to be determined and is depicted in their likely configuration. These compounds interact with cells in the local microenvironment, inhibiting PMN recruitment. COX-2-NSAID-dependent hydrogen abstraction and insertion of molecular oxygen with all ω-w PUFA containing 1, 4-cis pentadiene units.

Please replace the paragraph on page 12 beginning at line 29 with the following amended paragraph.

Figures 14A-14B depicts the DHA product profile switch triggered via acetylation of COX-2 by aspirin. The LC/MS ion chromatograms in the left panels show that, in the absence of aspirin, 13-hydroxy-DHA is the dominant product. With acetylation of COX-2 by aspirin, 13-hydroxy-DHA generation is suppressed and, shown in the right panel, 17-hydroxy-DHA generation is becomes the major product.

Please replace the paragraphs on page 13 beginning at line 6 through line 28 with the following amended paragraphs.

Figures 14~~B~~C-14D depicts an LC/MS/MS ion chromatogram and mass spectral analysis of 13-hydroxy-DHA generated from DHA by aspirin acetylated-COX-2.

Figures 14~~C~~E-14F depicts an LC/MS/MS ion chromatogram and mass spectral analysis of 14-hydroxy-DHA generated from DHA by aspirin acetylated-COX-2.

Figures 14~~D~~G-14H depicts an LC/MS/MS ion chromatogram and mass spectral analysis of 16-hydroxy-DHA generated from DHA by aspiring acetylated-COX-2.

Figures 14~~E~~I-14J depicts and LC/MS/MS ion chromatogram and mass spectral analysis of 17-hydroxy-DHA generated from DHA by aspirin acetylated-COX-2.

Figures 14~~F~~K-14L depicts an LC/MS/MS ion chromatogram and mass spectral analysis of 19-hydroxy-DHA generated from DHA by aspirin acetylated-COX-2.

Figures 14~~G~~M-14N depicts an LC/MS/MS ion chromatogram and mass spectral analysis of 20-hydroxy-DHA generated from DHA by aspirin acetylated-COX-2.

Please replace the paragraph on page 32 beginning at line 22 with the following amended paragraph.

Because ASA triggers formation of epimeric forms of naturally occurring bioactive eicosanoids (Reference 9), the concept that NSAIDs might promote the formation of novel mediators from ω-3 PUFAs was tested. Inflammatory exudates formed in murine air pouches via intrapouch injections of TNF-α with ω-3 and ASA on board (2 h) generated several novel compounds (Fig. 4). These mice were fed a standard rodent diet containing 0.26% ω-3 PUFA. LC/MS/MS analyses of the exudate-derived-materials demonstrated monohydroxy acids, depicted in selected ion chromatograms from acquired results recalled at m/z 317 (Fig. 4A), i.e., 18-hydroxy-EPA (18-HEPE) and 5-HEPE, which coeluted with synthetic 5*S*-HEPE as well as novel trihydroxy-containing compounds derived from C20:5. LC retention times and MS/MS spectra (Fig. 4 B and 4C) gave product ions consistent with structures shown in the respective insets, namely m/z 317 = (M-H)~-H_2O-CO_2. Diagnostic ions consistent with 18-HEPE identification were present at m/z 259 (Fig.4B) and 5-HEPE at m/z 115 (Fig. 4C). These criteria were used throughout for identification. The stereochemistry of the alcohol at carbon 18 was established for exudate-derived 18-HEPE using a chiral column, and a reference 18*R*-HEPE was

prepared via biogenic synthesis using *B. megaterium* (see Figures 5A-5C, Materials and Methods). This microbe monoxygenates fatty acids and, for example, converts C20:4 to 18*R*-HETE (22. Capdevila, J.H., S. Wei, C. Helvig, J.R. Falck, Y. Belosludtsev, G. Truan, S.E. Graham-Lorence, and J.A. Peterson. 1996. The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *J. Biol. Chem.* 271:22663-22671; and 23. Ruettinger, R.T., and A.J. Fulco. 1981. Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from *Bacillus megaterium. J. Biol. Chem.* 256:5728-5734.). The alcohol configuration at position 18 proved to be >98% *R*. These findings indicated that murine inflammatory exudates exposed in vivo to C20:5, ω-3 and ASA produced 5-lipoxygenase pathway 5-series 5*S*-HEPE, a product also identified with human PMNs, as well as a novel 18*R*-HEPE, whose route of formation was determined (vide infra) (24. Lee, T.H., J.M. Menica-Huerta, C. Shih, E.J. Corey, R.A. Lewis, and K.F. Austen. 1984. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene B5 and the double lipoxygenase product. *J. Biol. Chem.* 259:2383-2389.). Air pouch inflammatory exudate cells from these ASA- and EPA-treated mice contained predominantly PMN (as in Fig. 4), which were 25-50% lower in number than in exudates formed with TNF-α alone (n=3 illustrated in Fig. 6). Also, these exudates, when activated with ionophore A_{23187} (4 μM), generated essentially equivalent amounts of 18*R*-HEPE (10.2 ± 4.3 ng/10^4 cells) and 5*S*-HEPE (10.9 ± 2.9 ng/10^4 cells). Figures 4A-D depict inflammatory exudates from Murine Dorsal Pouches treated with Aspirin to generate novel compounds shown by LC/MS/MS; TNFα-induced leukocyte exudates were collected at 6 h from FVB mice given ASA (3.5 h at 500 µg/air pouch) and EPA (4 h at 300 µg/pouch), contained 2.3 +/- 0.5 X 10^6 leukocyts/pouch) (See Methods);

Please replace the paragraph on page 34 beginning at line 10 with the following amended paragraph.

It was of interest to determine whether these new compounds were also generated by human cells and if they possess bioactivities. To this end, human ECs known to induce COX-2 with IL-1β or hypoxia (not shown) were pulsed with EPA and treated with ASA, and extracted materials were subject to LC/MS/MS analysis (Fig. 7A). Selected ion monitoring at m/z 259 revealed that HUVECs treated with ASA converted EPA to 18*R*-HEPE (Fig. 7A). Also,

HMVECs created with ASA and EPA generated 18-HEPE (10.6 ng/10^4 cells) and 15-HEPE (6.0 ng/10^6 cells) (n = 2, four determinations; data now shown). These observations implicated the involvement of COX-2 in the generation of these compounds, which proved to be the case with recombinant human COX-2 exposure to ASA and ω-3 PUFA (Figs. 8A-8D and Table 1), findings that are of clinical significance.

Please replace the paragraph on page 34 beginning at line 21 with the following amended paragraph.

As shown in Table 1, linoleic acid (C18:2) was converted to both 13-hydroxy-9Z, 11E-octadocadienoic acid (13-HODE; n-5 oxygenation) and 9-HODE (ω-9), which were greatly diminished by ASA but not completely abolished. AA was converted to 15*R*-HETE (n-5) as well as 11*R*-HETE (n-9), consistent with earlier findings. ASA triggered the appearance of lipoxygenase activity that switched to 15*R*-HETE production by acetylated COX-2 (References 9, 14, 15), which did not appear to influence 11*R*-HETE formation (Table 1 and Fig. 8). 11*R*-HEPE was the major product with EPA and COX-2, with lesser amounts of 15*R*-HEPE (n-5) and 18*R*-HEPE (n-2). 1-^{14}C-labeled EPA was used to confirm precursor-product relationships (n = 3 as in Fig. 2 in Methods). ASA acetylation of COX-2 (Figs. 8A-8D for identification of each of the novel reaction products and indicated within their respective mass spectrum) led to an approximately twofold increase in 18*R*-HEPE (n-2), with a >85% reduction in 11*R*-HEPE (the ratio of positional oxygenation with C20:5 was 1:1:0.3, with 18*R* ~ 15*R* > 11*R*). Hence, together they suggested that acetylated COX-2 in ECs (Fig. 7) was a dominant source of 18*R*-HEPE and 15*R*-HEPE.

Please replace the paragraph on page 36 beginning at line 1 with the following amended paragraph.

This product is an 18*R*-hydroxy-carrying "LTB_5-like" structure (see Fig. 7 D, inset). Indeed, when isolated 18*R*-HEPE was incubated as above with activated PMNs, it was converted to several compounds, including this product. Also, synthetic LTB_5 incubated with *B. megaterium* homogenate and NADPH at pH 8.0 to facilitate hydroxylations (Reference 23) was transformed to a trihydroxy product (n = 3) with an m/z 291 ion characteristic for the presence of the 18R alcohol group (Figs. 5A-5C) as obtained from human PMNs show in Fig. 7 C. These

independent lines of evidence indicated that PMNs take up 18*R*-HEPE, which is converted by their 5-lipoxygenase, to insert molecular oxygen and in subsequent steps to 5-hydro(peroxy)-18*R*-DiH(p)EPE and 5(6)epoxide formation to 5, 12, 18*R*-triHEPE (an 18*R*-carrying LTB_5-like product) that is likely to posses the stereochemistry of LTB_5, retaining the 18*R* chirality of the precursor.

Please replace the paragraph on page 36 beginning at line 25 with the following amended paragraph.

Transendothielial migration is a pivotal event in PMN recruitment and inflammation and a recognized locus of action for traditional antiinflammatory therapies (27. Cronstein, B.N., S.C. Kimmel, R.I. Levin, F. Martiniuk, and G. Weissmann. 1992. A mechanism for the antiinflammatory effects of corticosteroids: The glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci.* USA 89:9991-9995.). Endogenous lipid mediators that can control these cell-cell interactions are of interest. Therefore, 5,12, 18*R*-triHEPE and in precursor 18*R*-HEPE on human PMN transmigration were evaluated and assessed. Both compounds inhibited LTB_4-stimulated PMN transendothelial migration (Fig. 9A) with an apparent IC_{50} for 5-50 nM for 5,12, 18R-triHEPE and $IC_{50} > 1.0\ \mu M$ for 18*R*-HEPE. Thus, the new 5-series members, namely, 18*R*-carrying trihydroxy-HEPE and 18*R*-HEPE, inhibited PMN migration, as did 15-epi-LXA_4, and in omega and analogue, tested in parallel for direct comparison (Figs. ~~10-A~~10A-10L, Table 1 and Table 2). Their rank order of potency was 15-epi-LXA_4 stable analogue > 5,12, 18*R*-tri-HEPE > 18*R*-HEPE.

Please replace the paragraph on page 37 beginning at line 12 with the following amended paragraph.

The G protein-coupled receptor for LTB_4 was identified (28. Yokomizo, T., T. Izumi, K. Chang, T. Takuwa, and T. Shimizu. 1997. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* 387:620-624.), and to determine whether these 18*R*-containing products interact with human LTB_4 receptors to block PMNs, this receptor was cloned (Reference 11) from reported sequences and stably expressed in HEK293 cells for competition binding experiments (Figs. ~~10-B~~10A-10L). The homoligand LTB_4, effectively competed (IC_{50} ~

2.5 nM). 18*R*-HEPE did not, while both LTB_5 and 5, 12, 18*R*-triHEPE competed (IC_{50} 0.5 μM), giving a trend with LTB_5 > 5, 12, 18*R*-triHEPE.

Please replace the paragraph on page 37 beginning at line 29 with the following amended paragraph.

When administered intravenously into tail at low levels (100 ng), 5, 12, 18*R*-triHEPE was a potent inhibitor of PMN infiltration into murine dorsal air pouches (Figs. ~~10 C~~10A-10L), as was a 15-epi-LX stable analogue given at equivalent doses for the purposes of direct comparison. 18*R*-HEPE also carried some activity in vivo (<5, 12, 18*R*-triHEPE), whereas it was far less effective with isolated human PMNs in transendothelial migration and apparently did not interact with recombinant LTB_4 receptors at these concentrations.

Please replace the paragraph on page 38 beginning at line 7 with the following amended paragraph.

Other widely used NSAIDs (i.e., acetaminophen and indomethacin) were also tested (Table 2) with recombinant COX-2 and C20:5 as in Table 1 to determine whether they altered conversion to HEPE. Each inhibited 11-HEPE by >95%. Interestingly, 18*R*-HEPE and 15*R*-HEPE formation persisted (~1:1 ratio) in the presence of either acetaminophen or indomethacin as concentrations as high as 2 mM, even though the levels of 15*R*- and 18*R*-HEPE were reduced by three to eight times their levels in the absence of inhibitors (n = 3). These findings indicate that the oxygenation of ω3 fatty acids to *R*-containing monohydro(peroxy)-containing products is not restricted to ASA treatment and arachidonate. Indeed, C18:2, C18:3 and C22:6 were also convereted by NSAID-COX-2 complexes to novel reaction products (See Figures 11A, B ~~and~~ ~~C~~C and D). Hence, these commonly used NSAIDs and selective COX-2 inhibitors (Table 1 and Table 2) still permit PUFA oxygenation by activated ECs exposed to NSAIDs (Fig. 7) and at sites of inflammation where the degree of COX-2 interactions with drugs to permit the generation of novel oxygenated forms of PUFAs.

Please replace the paragraph on page 38 beginning at line 21 with the following amended paragraph.

Despite the reports of ω-3 PUFAs (i.e., C20:5) possible beneficial impact in humans (References 1-6), oxygenation by COX-2 to generate novel bioactive compounds that has not been addressed in humans or isolated cells. In fish, both C20:5 and C20:4 are mobilized in macrophages and platelets to produce 5- and 4-series eicosanoids including PG, LT, and LX, with essentially equal abundance (Reference 26). The present invention provides that inflammatory exudates from mice treated with ASA and EPA generate novel compounds (Fig. 4) that are also produced by human ECs, recombinant COX-2, and PMNs (Figs. 5A-5C). Given the milligram to gram amounts of ω-3 PUFA taken as dietary supplements (References 1-6) and the large area of microvasculature that can carry upregulated COX-2, the conversion of EPA by ECs and neighboring cells as observed in the present experiments (Figs. 4-8) represent a significant amount at local microenvironments. These COX-2-NSAID-dependent conversions of ω-3 PUFA are likely to be elevated within inflamed or diseased tissues where COX-2 is upregulated and a determinant that impacts fatty acid metabolism when NSAIDs is of therapeutic benefit, namely with microinflammation.

Please replace the paragraph on page 39 beginning at line 7 with the following amended paragraph.

Analogous to 15-epi LX biosynthesis, EPA COX-2-derived 15*R*-HEPE was converted by 5-lipoxygenation with 5(6)-epoxied formation in leukocytes to give the 15-epi-LX_5 series (Figs. 11A-11D). The stable analogues of 15-epi-LXA_4, modified at their C15 position through position 20 with bulk groups, resist inactivating enzymes and are more potent in vivo, inhibiting PMN traffic as well as formation and actions of key proinflammatory cytokines (References 10 and 16). Hence, 5-series 15-epi-LXs should act in a similar fashion, as they possess a ∇17-18 double bond and thus could function as an ω-3-derived 15-epi-LX analogue.

Please replace the paragraph on page 39 beginning at line 16 with the following amended paragraph.

Because COX-2-NSAID-dependent oxygenation (e.g., 18*R* and 15*R*) led to bioactive compounds in vivo that block PMN transendothelial migration and infiltration, the findings provide a basis for novel mechanism of action of NSAIDs and dietary ω-3 supplementation, namely the generation of endogenous functional arrays of lipid signals (Table 1 and Figs. 4 and 7) that could, by dampening key events in microinflammation, mediate some of the beneficial actions noted for ω-3 therapies in human trials. In this context, 13-HODE, a recognized lipoxygenase product that downregulates platelet-EC interactions (29. Buchanan, M.R., P. Horsewood, and S.J. Brister. 1998. Regulation of endothelial cell and platelet receptor-ligand binding by the 12- and 15-lipoxygenase monohydroxides, 12-, 15-HETE and 13-HODE. *Prostaglandins Leukot. Essent. Fatty Acids* 58:339-346.), is also generated by COX-2 (Table 1 and 2) and joins as does DHA (C22:6) (Figs. ~~10C~~10A-10L) this pathway array and class of mediators (Fig. 12). DHA was also converted to novel reaction products (Fig. 13). In addition, ASA treatment of COX-2 gave different ratios of C17 versus C13 products (Figs. 14 ~~B-G~~A-N). These new COX-2 products are likely to play roles in the brain vasculature where COX-2 and DHA are elevated. Hence these compounds could be used to treat inflammatory disorders in neuronal tissues (i.e., Parkinson's disease, Alzheimer's disease, etc.). Hence, it has been surprisingly discovered that COX-2 interactions with NSAIDS lead to novel oxygenations of a wide range of lipid precursors to produce bioactive compounds as illustrated in Fig. 15 and can be used in therapeutic treatment.